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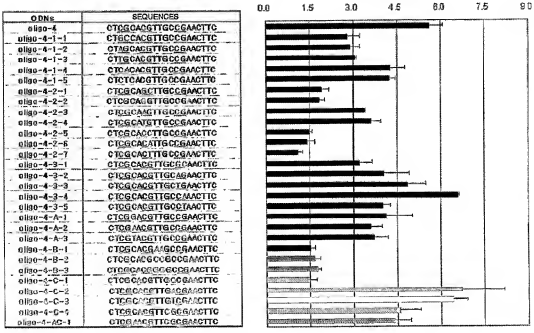

(57) **Abstract:** Oligonucleotides for stimulating immune response are disclosed. The oligonucleotides may be used as an immune stimulator (or an adjuvant), and may be used for immune response rebalance.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

OLIGONUCLEOTIDES FOR STIMULATING IMMUNE RESPONSE

Technical Field

The present invention relates to oligonucleotides for stimulating immune response.

Background Art

In general, vertebrate animals have evolved the immune system that recognizes a few characteristic microbial molecules to rapidly perform immune activity against microbial invasion. Several investigators reported that bacterial DNA has characteristic motifs that are not common in vertebrate DNA. These factors have been found to activate immune cells (Gilkeson, G. S. et al. (1995) *J. Clin. Invest.* 95, 1398-1402). Distinguished differences between vertebrate DNA and bacterial DNA are in that vertebrate DNA is CpG suppressed and approximately 70% of the vertebrate DNA is methylated to cytosine of CpG motif dinucleotide (Krieg, A. M., et al. (1995) *Nature* 374, 546-549. Here, CpG suppression means that about 30 to 50% CpG fewer than statistically expected are expressed. CpG suppression has been reported to be expressed in intracellular parasites such as Plasmodium falciparun, and bacterial genomes such as Mycobacterium jannaschii. When the intracellular parasites and bacteria invade vertebrates, they act like vertebrate genomes due to CpG suppression, thereby inducing immune avoidance (Karlin, S., et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 837-841). There has been research into CpG motif that is most contributable to activation of innate immune system. According to the

results, in mice and rabbits, the sequence of purine-purine-CG-pyrimidine-pyrimidine is the greatest contributing sequence in increasing immunoactivity, most preferably GACGTT (Yi. A. -K., et al. (1998) *J. Immunol.* 160, 5898-5906). On the other hand, it has been known that GTCGTT is the most optimal CpG motif in humans (Hartmann, G., et al. (2000) *J. Immunol.* 164, 944-953). It has been demonstrated that GTCGTT, which is an optimal CpG motif in humans, is also optimal CpG motif in vertebrate animals such as cows, sheep, cats, dogs, goats, horses, pigs, or chickens. (Brown, W. C., et al. (1998) *Infect. Immun.* 66, 5423 - 5432). More recently, it has also been found that a CpG motif expressed by bacterial DNA rapidly activates polyclonal B cell to promote secretion of IgM (Yi, A. -K., et al.) and CpG motifs directly activates B cell to rapidly secrete IL-6 and IL-12 (Yamamoto, S., et al. (1992) *Microbiol. Immunol.* 36, 983-997). Also, it has been found that CpG motif acts on NK cells to induce IFN-γ in CD4+ cell (Stacey, K. J., et al. (1996) *J. Immunol.* 156, 4570-4575).

Many researchers have demonstrated that CpG motif expressed by *E. coli* chromosomal DNA (to be referred to as "EC DNA" hereinafter) activates the innate immune response (Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. (1996) *J. Immunol.* 157, 1840-1845). However, since the EC DNA is large-sized and has a complex structure, it cannot directly act on an immune cell when CpG motif is not exposed to the surface thereof.

Disclosure of the Invention

To solve the above problems, it is an object of the present invention to provide oligonucleotides for stimulating immune responses by partially digesting

large-sized EC DNA by DNase I and to provide determining optimal sequences by modification of sequences.

To accomplish the above object of the present invention, there is provided oligonucleotides for stimulating immune response, comprising a sequence of 5'-RYCGYRCGYYG[R/Y]CRRR-3'(SEQ ID NO: 1), where R represents purine nucleotides such as adenine or guanine, and Y represents pyrimidine nucleotides such as cytosine or thymine.

Also, the present invention provides oligonucleotides for stimulating immune response further comprising YYYY at their 3' terminal. Here, Y represents pyrimidine nucleotides such as cytosine or thymine.

In the present invention, the oligonucleotides preferably comprises a sequence of 5'-GTCGCACGTTGACGAA-3' (SEQ ID NO: 8), 5'-GTCGCACGTTGTCGAA-3' (SEQ ID NO: 9) or 5'-GTCGCACGTTGCCAAA-3' (SEQ ID NO: 10), more preferably 5'-GTCGCACGTTGCCGAA-3' (SEQ ID NO: 2), and most preferably 5'-GTCGCACGTTGACGAACTTC-3' (SEQ ID NO: 11), 5'-GTCGCACGTTGTCGAACTTC-3' (SEQ ID NO: 12), 5'-GTCGCACGTTGCCAAACTTC-3' (SEQ ID NO: 13) or 5'-GTCGCACGTTGCCGAACTTC-3' (SEQ ID NO: 3).

In the present invention, the nucleotide-nucleotide bond can be both a phosphodiester bond and a phosphorothioate bond. In a preferred embodiment, the nucleotide-nucleotide bond is a phosphodiester bond.

In an embodiment, the respective linked sites of the nucleotides are all substituted by phosphorothioate bonds. Here, non-bridging oxygen is substituted with sulfur. This technique is often employed by Antisense RNA

researchers, and is available from most synthetic oligo manufactures. All synthetic oligonucleotides used in the present invention are purchased from Geno Tech Corp. (Daejeon-city, Korea).

Brief Description of the Drawings

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention, in which:

FIG. 1 shows IL-8 promoter activation of EC DNA treated RAW 264.7 cells. FIG. 1a shows the comparison results of the level of IL-8 promoter activation of macrophages depending on the sizes of EC DNA fragments, and FIG. 1b shows immune response induction experiment of macrophages depending on the treatment time of EC DNA fragments;

FIG. 2 shows IL-12 promoter activation of EC DNA treated RAW 264.7 cells of various sizes for 24 hours, in which EC DNA1 is intact *E. coli* chromosomal DNA, EC DNA2 > 1 Kbp, EC DNA3 is in the range of 100 bp~1 Kbp, EC DNA4 is in the range of 50~200 bp, and EC DNA5 is in the range of 10~50 bp;

FIG. 3 shows the humoral immune responses of Balb/c mice peritoneally immunized by EC DNA and hen egg lysozyme (HEL);

FIG. 4 shows the nuclear localization of NF-κB according to EC DNA stimulation, in which Raw 264.7 cells are treated with EC DNA4 (1.5 μg/ml) for 20 minutes to fix the cells, indirect immunofluorescence is performed with NF-

κB p65-specific antisera, and staining is performed with Hoechst NO.33258 to visualize the nuclei;

FIG. 5 shows the results of IL-8 promoter luciferase assay with synthetic oligonucleotides, in which FIG. 5a shows screening of synthetic nucleotides by luciferase assay using promoter-reporter constructs, FIG. 5b shows oligo-4 of 5'-GTCGCACGTTGCCGAACTTC-3', oligo-4S as its phosphorothioester type, and oligo-4short of 5'-GTCGCACGTTGCCGAA-3'; and

FIG. 6 shows the results of IL-8 promoter luciferase assay with sequence-modified oligo-4; and

FIG. 7 shows comparison results of luciferase assay effect exhibited when a phosphothioate backbone is modified in the sequence linkage structure of oligo-4 and control group, in which FIG. 7a shows the luciferase assay effect exerted on phosphodiester backbone of oligo-4 in the RAW 264.7 cell line, and FIG. 7b shows the luciferase assay effect exerted on phosphodiester backbone of oligo-4 in the RPMI 8226 cell line;

FIG. 8 shows comparison results of cytokine production by treatment of human peripheral blood mononuclear cells (PBMC) with oligo-4 and control group with oligo, in which FIG. 8a shows that IL-12 p40 production is increased by phosphodiester type oligo-4 in the PBMC, and FIG. 8b shows that IFN-γ production is increased by phosphodiester type oligo-4 in the PBMC;

FIG. 9 shows comparison results of expression of cytokines associated with inflammatory responses when RAW 264.7 cell line is treated with various kinds of oligos, confirming that treatment with phosphodiester type oligo-4 increases IL-12 proportion.

Best mode for carrying out the Invention

Hereinafter, the present invention will be described in more detail with reference to non-limiting embodiments.

Example 1: Isolation of *E. coli* chromosomal DNA (EC DNA)

E. coli was cultured in a shaking incubator, the cultured E. coli was recovered by centrigugation (yield: 15 g), and washed. Then, lysis A solution (Tris- EDTA pH 7.4, 100 μg/ml lysozyme) was added to the resultant material and reacted. After one-hour reaction, a lysis B solution (Tris-EDTA pH 7.4, 1% SDS, 25 μg/ml RNase A) was added to the reactant product and reacted for one hour at 37°C. Proteinase K (100 μg/ml) was added and reacted for 4 hours, followed by homogenizing E. coli. Then, the supernatant was extracted by centrifugation. Chloroform and the supernatant are added in the same amount and reacted for 4 hours. The supernatant was extracted by centrifugation, and EC DNA (3 mg/ml) was obtained by ethanol precipitation.

Example 2: Production of EC DNA Fragments using Enzymatic Activity of DNase I and Removal of Endotoxin

Chromosomal DNA derived from E. coli was digested by the enzymatic activity of bovine DNase I, thereby producing EC DNA fragments. 200 mg of EC DNA and 0.02 units of DNase I were reacted at 37°C for 0 minute, 5 minutes, 10 minutes, 40 minutes, and 2 hours. 10 ml of phenol, chloroform and isoamylalcohol were mixed in a ratio of 25:24:1 and centrifuged to extract

supernatant. Then, DNA was precipitated by ethanol precipitation, and the precipitate was washed with distilled water and dried. After the precipitate was dissolved in distilled, Triton X-114 was added to reach the final concentration of 0.5%, and reacted at 4°C for 4 hours. The resultant product was allowed to stand at 37°C for 5 minutes and centrifuged at room temperature to extract supernatant. DNA was precipitated by ethanol precipitation, and the precipitate was washed with 70% ethanol to dry the precipitate. The precipitate was dissolved in 200 ml of distilled water, producing EC DNA fragments.

In order to remove LPS, the produced fragments were treated with triton X-114 and reacted at 4°C for 4 hours. In such a manner, LPS was dissolved by a detergent layer and supernatant was then isolated. It was confirmed that the LPS contained in each EC DNA fragment sample was measured by Limulus Amebocyte Lysate (LAL) test kit for measuring the amount of endotoxin (manufactured by BioWhittaker), confirming the level of the LPS detected was 0.075 EU/ml or less. The EC DNA fragment samples, from which LPS was removed, were used in carrying out experiments in the present invention.

Example 3: Immune Response Induction of EC DNA fragments

1) Macrophage cell line culture of mice

RAW 264.7 cells as macrophage cell lines of mice were purchased from American Type Culture Collection ([ATCC] Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (manufactured by Gibco BRL). The cells were cultured in an incubator containing 5% CO_2 (Forma) at 37°C. The number and survival rate

of cells while the cells are cultured were periodically measured using a hemocytometer by trypan blue exclusion. Throughout the experiments, the survival rate of cells was maintained at higher than 95%.

2) Luciferase reporter plasmid

IL-8 promoter portions corresponding to -135 bp to +146 bp were PCR-amplified on human genomic DNA (Clontech) using primers shown in Table 1.

Table 1

5' primer (SEQ ID NO: 4)	5'GTGAGATCTGAAGTGTGATGACTCAGG3'
3' primer (SEQ ID NO: 5)	5'GTGAAGCTTGAAGCTTGTGTGCTCTGC3'

pGL3-Basic plasmid (Promega) was cleaved with restriction enzymes *Bgl* II and *Hind* III, and the amplified IL-8 promoter portions were linked to prepare IL-8-*Luc* reporter constructs (Wu, G. D., et al. (1997) *J. Biol. Chem.* 272, 2396-2403). IL-12 promoter portions ranging from -373 bp to +52 bp were PCR-amplified on human genomic DNA (Clontech) using primers shown in Table 2.

Table 2

5' primer (SEQ ID NO: 6)	5'CATGAGCTCAGCCTCCCGTCTGACC3'
3' primer (SEQ ID NO: 7)	5'CTGGGCTCGAGGGAGAGTCCAATGG3'

pGL3-Basic plasmid (Promega) was cleaved with restriction enzymes

Sac I and *Xho* I, and amplified IL-12 promoter portions were liked to product IL-12-*Luc*reporter constructs (Plevy, S., et al. (1997) *Mol. Cell. Biol.*17, 4572 - 4588).

3) Transfection

RAW 264.7 cells were placed in a 12-well plate in an amount of 5×10^5 cells/well and cultured at 37°C for 24 hours. The culture medium was removed and washed with PBS. 1 ml of fresh DMEM culture medium was added to each well and cultured again at 37°C for one hour. FuGene 6 transfection reagent (Roche) (1.5 μl/well), luciferase-reporter plasmid (0.2 μg/well), pRL-null plasmid (20 ng/well) and serum free DMEM (50 μl/well) were mixed. Reporter plasmid was transfected to RAW 264.7 cells and cultured at 37°C for 24 hours.

4) Identification of IL-8, IL-12 Promoter Activation by EC DNA Fragments

EC DNA fragments of 50-200 bp (3 μg/well) were distributed to each well to treat IL-8 promoter reporter construct-transfected RAW 264.7 cells and cultured at 37°C for various time periods of 2, 4, 6, 8, and 12 hours. The culture medium was removed and a passive lysis buffer of Dual-Luciferase reporter assay system (Promega) was added to each well (100 μl/well) to pulverize the RAW 264.7 cells. The cell lysates were centrifuged to remove the cell debris. 15 μl of the obtained supernatant was subjected to luciferase assay. Luciferase activity was measured using a TD-20/20 (Turner designs) luminometer. In the same manner, EC DNA fragments were treated at various sizes (3 μg/well) and reacted in a 5% CO_2 incubator at 37°C for 6 hours to evaluate IL-8 promoter

activation. In the IL-12 promoter activation experiment, after treating the EC DNA fragments, the reaction was maintained for 24 hours and then the promoter activation was evaluated.

In order to identify immune response induction of macrophage cells by EC DNA fragment size, the activation of a promoter (IL-8 promoter) containing NF-κB binding sequence was evaluated by luciferase assay. As shown in FIG. 1a, the IL-8 promoter activation levels of the macrophage cells depending on the EC DNA fragment size were compared. The comparison results showed that the promoter activation level was higher as the size is reduced. In the immune response induction experiment of the macrophage cells depending on the treatment time EC DNA fragments, as shown in FIG. 1a, it was confirmed that the IL-8 promoter activation level was highest when the EC DNA fragments were treated for 8 hours. FIG. 2 shows the experimental results of promoter activation of macrophage cells using IL-12 p40 promoter in the same manner as described above. Like in the IL-8 promoter activation experiment, as the size of EC DNA fragment was reduced, the level of activation increased.

Example 4: Induction of humoral immune response of EC DNA fragments

1) Immunization

A mixture of hen egg lysozyme (HEL, 50μg/mouse) and EC DNA fragment of various sizes (100 μg/mouse) was intraperitoneally administered to 4-week Balb/c mice. After 1 week, the second administration of the same amount of the mixture was performed. At the end of one week, the mice were bled by heart punching, and centrifugation was performed to precipitate blood

cells, thereby obtaining mouse serum. ELISA was performed on the serum to measure the titer of anti-HEL antibody (total IgG).

2) ELISA

The serum was diluted with PBS/0.2% sodium azide in a ratio of 1:10 and stored at -20°C. 10 μg/ml of HEL was placed in a 96 well Immunoplate (Nunc), allowed to stand at 4°C for 16 hours, and plated on the bottom of the plate. The plate was washed with PBST containing PBS and 0.05% Tween 20, 1% BSA was added thereto for blocking, and allowed to stand at room temperature for one hour. The serum was continuously 3-fold diluted with PBS plate to then be added to the plate, allowed to stand at 4°C for 16 hours, and washed with PBST. Alkaline phosphatase-conjugated detecting antibody was mixed with PBST and added to the plate, followed by allowing the plate to stand at room temperature for 2 hours. 1:2,000 goat anti-mouse Ig(H+L) (Southern Biotechnology Associates) antibody was used to detect the total Ig. To achieve color development, 1-stepTM ABTS (Pierce Chemical) was added to the resultant product, and the absorbance was measured with ELISA reader (Labsystems) at 405 nm.

EC DNA fragments were administered into the peritoneal cavity of mice with hen egg lysozyme (HEL) to examine humoral immune responses. When EC DNA fragments of 100 bp to 1 kbp were administered together, the IgG titer was highest (FIG. 3). Compared to the case where HEL was administered alone, the amount of the antibody in the case where HEL was administered together with the EC DNA fragments increased, suggesting that the EC DNA fragments

effectively acted as an immune adjuvant in the humoral immune responses. Freund's adjuvant is one of the representative immune adjuvants that have been widely used until now. However, this immune adjuvant has a problem in that it is incapable of stimulating cellular immune responses. It was found EC DNA to act not only as an immune adjuvant for stimulating humoral immune responses but also as an immune cell stimulator for inducing cellular immune responses, In conclusion, EC DNA fragments can be effectively used as a new immune adjuvant.

Example 5: NF-κB Activation by EC DNA Fragment Treatment

A cover glass was placed in a 24-well plate, Raw 264.7 cells were added thereto in an amount of 5×10^5 cells/ml and cultured at 37°C in a 5% CO_2 incubator for 24 hours. Each 1.5 μg of EC DNA fragments of 50-200 bp was treated for each well. After 20 minutes, to fix the cells, PBS containing 3.7% formaldehyde was added to 200 μl in each well and allowed to stand at room temperature for 10 minutes, followed by washing with PBS. In order to make the cells permeable, PBS containing 0.2% Triton-X 100 was added to 200 μl in each well and allowed to stand at room temperature for 10 minutes, followed by washing again with PBS. A solution obtained by adding 1% donkey serum to PBS (PBST) containing 0.2% Tween 20 was placed in each well (200 μl/well) and blocked for 30 minutes. Then, mouse anti-p65 (titer 1: 500) antibody (0.5 μl/well) was added to PBST and allowed to stand at room temperature for 2 hours, followed by washing the well (200 μl/well) with PBST. The same procedure was repeated 3 to 4 times. 0.5 μl of donkey-anti-mouse-IgG-FITC

(titer 1:250) antibody was added to each well and allowed to stand for 2 hours. The resultant product was repeatedly washed with PBST 3 to 4 times and dried, followed by observing nuclear localization of NF-κB using confocal microscopy.

FIG. 4 shows the nuclear localization of NF-κB by immunostatining, as observed by confocal microscopy. In control group to which no treatment has been applied, NF-κB was sequestered in cytoplasm. However, when the cells were treated with LPS, it was confirmed that NF-κB localization toward the nucleus after 20 minutes. The same result was applied to the case where the macrophage cells were treated with EC DNA fragments, that is, NF-κB was localized to the nucleus.

Example 6: Sequence Analysis of EC DNA Fragments

1) Enzyme treatment of EC DNA fragments

EC DNA fragments of 100-1000bp were subjected to ethanol precipitation, and the precipitate was dissolved in distilled water. 10 mM of Tris-HCl, 5 mM of $MgCl_2$, 7.5 mM of dithiothreitol (pH 7.5, 25°C), 1 mM dNTP, and 5 units of Klenow (NEB), and 10 μg of EC DNA were reacted at 25°C for 20 minutes in the presence of a Klenow enzyme, thereby achieving gap filling. After 20 minutes, the reaction was terminated, and EC DNA fragments were recovered by nucleotide removal kit (Qiagen). In order to phosphrylate EC DNA, 70 mM of Tris-HCl, 10 mM of $MgCl_2$, 5 mM of dithiothreitol (pH 7.6, 25°C), 10 units of T4 polynucleotide kinase (NEB), and 5 μg of EC DNA at 37°C were reacted for one hour in the presence of a polynucleotides kynase, and 1 μl of 0.5 M EDTA was added thereto, thereby terminating the reaction.

2) Enzyme treatment of pGEM-T easy vector

In order to cleave pGEM-T easy vector (Promega), 20 units of *EcoR*I (NEB) and 2.5 μg of pGEM-T easy vector (Promega) were reacted at 37°C for 2 hours. 0.5 M EDTA was added in an amount of 1 μl to terminate the reaction and vector recovery was made using a nucleotide removal kit (Qiagen). To achieve gap-filling, 10 mM of Tris-HCl, 5 mM of $MgCl_2$, 7.5 mM of dithiothreitol (pH 7.5, 25°C), 1 mM dNTP, 5 units of Klenow (NEB), and 1 μg of vector were reacted at 25°C for 20 minutes, and 1 μl of 0.5 M EDTA was added thereto to terminate the reaction. The vector was recovered using a nucleotide removal kit (Qiagen). In order to remove phosphates from the recovered vector, 100 mM of NaCl, 50 mM of tris-HCl, 10 mM of $MgCl_2$, 1 mM of dithiothreitol (pH 7.9, 25°C), 2.5 units of calf intestinal alkaline phosphatase (CIP), and 0.5 μg of vector was reacted at 37°C for one hour. The vector and EC DNA fragments were subjected to electrophoresis with 2% agarose gel to sever bands, respectively, and recovered using a gel extraction kit (Qiagen). In order to ligate the phosphate-removed vector with the phosphorylated EC DNA fragments, 50 mM of Tris-HCl, 10 mM of $MgCl_2$, 10 mM of DTT, 1 mM of ATP, 25 ug/ml of BSA (pH 7.5), 0.5 μg of vector, and EC DNA 2 μg were reacted in the presence of 400 units of T4 DNA ligase (NEB) and further reacted at 16°C for 16 hours. The resulting ligates were transformed using E. coli XL-1 blue competent cells synthesized by a $CaCl_2$ method, cultured at 37°C for 12 hours, and plated in a LB plate, thereby screening white colonies.

3) Confirmation of Insertion and Sequences

The white colonies were cultured in a liquid LB (50 mg/ml ampicillin), respectively, cultured, centrifuged to obtain E. coli, and finally obtaining plasmid DNA using a QIAprep Spin Miniprep kit (Qiagen). The obtained plasmid DNA was cleaved with reatriction enzymes *Nco* I (NEB) and *Pst* I (NEB), confirming inserted size. Sequences of EC DNA fragments were confirmed by DNA sequencing, and analyzed for listing 20 oligonucleotides.

Example 7: Examination of Immune Response of Synthetic Oligonucleotides

RAW 264.7 cells were plated in a 12-well plate (5×10^4 cell/well) and cultured in a 5% CO_2 incubator at 37°C for 24 hours. IL-8 promoter reporter plasmid and pRL-null plasmid were co-tranfected and cultured in a 5% CO_2 incubator at 37°C for 24 hours. Synthetic oligonucleotides were treated (10 μg/well), and tranfected, and cultured in a 5% CO_2 incubator at 37°C for 6 hours. Then, the culture medium was removed and a passive lysis buffer of Dual-Luciferase reporter assay system (Promega) was added to each well (100 μl/well) to pulverize the RAW 264.7 cells. The cell lysates were centrifuged to remove cell debris. 15 μl of the obtained supernatant was subjected to luciferase assay. Luciferase activity was measured using a TD-20/20 (Turner designs) luminometer. In the same manner, IL-8 promoter-reporter plasmid, pRL-null plasmid and IκBα supper repressor (IκBα SR) plasmid were co-transfected to evaluate immune responses of the synthetic oligonucleotides.

EC DNA fragments were cloned to pGEM-T-easy vector to determine the

immuno-stimulatory sequences. Sequences of 34,173 bp in full length were assayed for 117 DNA fragments of 100 to 800 bp. This corresponds to 0.7% of chromosomal DNA size of *E. coli* K-12 strains, i.e., 4,639,221 bp. These sequences were randomly restricted to 20 bases using a computer program, and sequences having two or more CpG motifs were listed. In order to identify which sequence is derived from the synthetic oligonucleotide associated with IL-8 promoter activation of macrophage cells, luciferase assay was performed to evaluate the activation level. The results demonstrated that the oligonucleotide having the sequence of 5'-GTCGCACGTTGCCGAACTTC-3' (oligo-4) exhibited more than 8 times a higher level of IL-8 promoter activation than the control group (FIG. 5A). Other synthetic oligonucleotides exhibited substantially no effect of IL-8 promoter activation, which is also applicable to the control group. 5'-GTCGCACGTTGCCGAA-3' was synthesized from the oligonucleotide that has activated the Il-8 promoter by removing four bases CTTC of 3' end, i.e., a non-CpG motif portion, and the IL-8 promoter activation level was then measured. The measurement result showed that the activation level of the newly synthesized product was about 10 times higher than that of the control group. Also, when phosphodiester bonds were modified to phosphorothioate bonds, the synthetic oligonecleotide showed a level of IL-8 promoter activation of about 11 times higher than the control group (FIG. 5B).

Example 8: Inspection of Sequence Modification and Immune Response of Synthetic Oligonucleotides

1) Sequence Modification of oligo-4

Sequences of oligo-4 were modified. The first CG sequence of oligo-4 was modified to GC, AG, TG, CA, and CT, and the second CG sequence was modified to GC, GG, AG, TG, CC, CA, and CT. The third CG sequence was modified to GC, AG, TG, CA, and CT. A base sequence of CA positioned between the first sequence CG and the second sequence CG was replaced by GA, AA, and TA. A base sequence of TTGC positioned between the second CG and the third CG was replaced by AAGC, CCGC, GGGC, TTGG, TTGA, TTGT, and TTCG.

2) Inspection of Immune Response of Sequence Modified oligo-4

Raw 264.7 cells were plated to a 12-well plate (5×10^4 cells/well) and cultured in a 5% CO_2 incubator at 37°C for 24 hours. IL-8 promoter- reporter plasmid and pRL-null plasmid were tranfected and cultured in a 5% CO_2 incubator at 37°C for 24 hours. Synthetic oligonucleotides were treated (10 μg/well), and cultured in a 5% CO_2 incubator at 37°C for 6 hours. The culture medium was removed and a passive lysis buffer of Dual-Luciferase reporter assay system (Promega) was added to each well (100 μl/well) to pulverize the RAW 264.7 cells. The cell lysates were centrifuged to remove cell debris. 15 μl of the obtained supernatant was subjected to luciferase assay. Luciferase activity was measured using a TD-20/20 (Turner designs) luminometer.

In order to identify which sequence is derived from the synthetic oligonucleotides associated with IL-8 promoter activation of macrophage cells, luciferase assay was performed to evaluate the activation level. The results demonstrated that the oligonucleotide having a sequence of 5'-

GTCGCACGTTGCCAAACTTC-3', 5'-GTCGCACGTTGACGAACTTC-3', or 5'-GTCGCACGTTGTCGAACTTC-3' exhibited a relatively high level of IL-8 promoter activation than the control group (FIG. 6). Other synthetic oligonucleotides exhibited a relatively low level of IL-8 promoter activation. Among oligonucleotides that have activated the Il-8 promoter, a variant of the third GCCGAA sequence, that is, GCCAAA, i.e., a non-CpG motif portion, also exhibited IL-8 promoter activation. Based on the fact that the sequence of 5'-GTCGCACGTTGACGAACTTC-3', or 5'-GTCGCACGTTGTCGAACTTC-3' is involved in IL-8 promoter activation, it could be concluded that the IL-8 promoter activation can be enhanced by modifying the sequence adjacent to the third CG sequence of oligo-4 (FIG. 6).

1826, 2006, and 2041 mentioned in examples that follow after Example 9 were oligonucleotides used as comparison examples. In detail, the sequence 1826 represents TCCATGACGTTCCTGACGTT, 2006 represents TCGTCGTTTTGTCGTTTTGTCGTT, and 2041 represents CTGGTCTTTCTGGTTTTTTTCTGG. (O) means a phosphodiester bond and (S) means a phosphorothioate bond.

Example 9: Comparison of IL-8 Promoter Activation by Oligos Modified in Phosphodiester and Phosphorothioate Backbones

IL-8 promoter-reporter construct transfected RAW 264.7 cells and RPMI 8226 cells were treated with each 10 μg/ml of oligos having phosphodiester and phosphorothioate backbones. After 8 hours, the culture medium was removed and a passive lysis buffer of Dual-Luciferase reporter assay system (Promega)

was added to each well (100 μl/well) to pulverize the RAW 264.7 cells. The cell lysates were centrifuged to remove cell debris. 15 μl of the obtained supernatant was subjected to luciferase assay. Luciferase activity was measured using a TD-20/20 (Turner designs) luminometer.

FIG. 7 shows that oligos exhibited different levels of IL-8 promoter activation according to the oligonucleotide sequence and backbone. As shown from FIG. 7, among phosphodiester type oligos, oligo-4 was most effective on the RAW 264.7 cell line, which is a mouse macrophage line. In the RPMI 8226 cell, which is a human B cell line, only phosphorothioate oligos activated the IL-8 promoter. In particular, oligo-4 exhibited the highest activation level.

Example 10: Comparison Cytokine Production Yields by Treating Human Leukocyte Cell with oligo-4 and oligo as Control Group

PBMC was collected from human peripheral blood and each 1×10^6 cells/ml of the collected PBMC was treated with oligos (10 μg/well) for 24 hours. The culture medium was isolated and subjected to sandwich ELISA. For production of cytokine, human IFN-γ Quantikine M reagent (R&D Systems, Minneapolis, Minn.) or human IL-12 p40 reagent (R&D Systems, Minneapolis, Minn.) was used. The experiment was performed twice and the obtained experimental data were averaged. Antibody against cytokine was diluted with a carbonate buffer (SIGMA. C-3041) and coated on a 96-Well plate (NUNC. 442404), followed by blocking with 3% BSA (SIGMA. A-2154). Samples to be tested were diluted at appropriate proportions and distributed to each well, followed by incubation for 2 hours. Then, the resultant product was reacted with

the antibody against biotinylated cytokine at 37°C for 1 hour and reacted with SaV-HRP (Pharmingen, 13047E) for 30 minutes, followed by washing. Color development was induced and absorbance was measured at 490nm.

FIG. 8a shows that IL-12 p40 production is increased by phosphodiester type oligo-4 in the human PBMC, and FIG. 8b shows that IFN-γ production is increased by phosphodiester type oligo-4 in the PBMC.

Example 11: Comparison of expressed cytokines associated with inflammatory responses when treated with various kinds of oligos

10 μg/ml of phosphodiester type oligo and 5 μg/ml of phosphorothioate type oligo 1826 were treated on RAW 264.7 cell line for 0.5, 1, 2, 4, 8, 12 hours. Total RNA was extracted from the cell using a MicroRNA Isolation Kit (Stratagene, La Jolla, CA). cDNAs were derived from 5 μg of total RNA using 50 units of StrataScript reverse transcriptase and oligo (dT) primers (Stratagene, La Jolla, CA). 2 μl (5 μg) of total RNA was reversely transcripted by first-strand cDNA specific primer. PCR amplification was performed by several steps, including DNA denaturation at 94°C for 1 minute, primer annealing at 60°C and DNA extension at 72°C for 2 minutes.

FIG. 9 shows comparison results of expression of cytokines associated with inflammatory responses when RAW 264.7 cell line is treated with various kinds of oligos, confirming that treatment with phosphodiester type oligo-4 increases an IL-12/TNF-alpha proportion.

Industrial Applicability

As described above, the EC DNA fragments according to the present invention act as an immune adjuvant in forming HEL antibody to be associated with humoral immune responses. In the course of conducting research into IL-8 promoter activation of macrophage cell, it was found that IL-8 promoter activation has brought about activation of innate immune cell. In particular, in order to identify specific sites inducing immune responses in EC DNA fragments, 20mer synthetic oligonucleotides were screened. Among the synthetic olidgonucleotides, the most effective DNA fragment sequences in IL-8 promoter activation of macrophage cell are 5'-GTCGCACGTTGACGAA-3', 5'-GTCGCACGTTGTCGAA-3', 5'-GTCGCACGTTGCCAAA-3' or 5'-GTCGCACGTTGCCGAA-3', and 5'-GTCGCACGTTGACGAACTTC-3' , 5'-GTCGCACGTTGTCGAACTTC-3', 5'-GTCGCACGTTGCCAAACTTC-3' or 5'-GTCGCACGTTGCCGAACTTC-3'.

Also, to evaluate effects of treatment with various kinds of oligos, expression levels of cytokines associated with inflammatory responses are compared. According to the comparison result, treatment with phosphodiester type oligo-4 increases IL-12/TNF-alpha proportion.

The present invention has been described above by reference to the particular embodiments and figures, which, however, should not be construed as limitations on the scope of the invention but merely as illustrations of some of the presently preferred embodiments. It is to be understood that other modifications or substitutions may be made to the invention at the time of filing of this invention without departing from the scope of the invention.

What is claimed is:

1. An oligonucleotide for stimulating immune response, comprising a sequence of 5'-RYCGYRCGYYG[R/Y]CRRR-3'(SEQ ID NO: 1), where R represents a purine nucleotide, including adenine and guanine, and Y represents a pyrimidine nucleotide, including cytosine and thymine.

2. The oligonucleotide of claim 1, further comprising YYYY at its 3' terminal, where Y represents a pyrimidine nucleotide, including cytosine and thymine.

3. The oligonucleotide of claim 1, comprising a sequence of 5'-GTCGCACGTTGACGAA-3' (SEQ ID NO: 8), 5'-GTCGCACGTTGTCGAA-3' (SEQ ID NO: 9), or 5'-GTCGCACGTTGCCAAA-3' (SEQ ID NO.: 10).

4. The oligonucleotide of claim 2, comprising a sequence of 5'-GTCGCACGTTGACGAACTTC-3' (SEQ ID NO: 11), 5'-GTCGCACGTTGTCGAACTTC-3' (SEQ ID NO: 12), or 5'-GTCGCACGTTGCCAAACTTC-3' (SEQ ID NO: 13).

5. The oligonucleotide of claim 1, comprising a sequence of 5'-GTCGCACGTTGCCGAA-3' (SEQ ID NO: 2).

6. The oligonucleotide of claim 2, comprising a sequence of

5'-GTCGCACGTTGCCGAACTTC-3' (SEQ ID NO: 3).

7. The oligonucleotide of claim 1 or 2, wherein the nucleotide-nucleotide bond is a phosphodiester bond or a phosphorothioate bond.

FIG. 1a

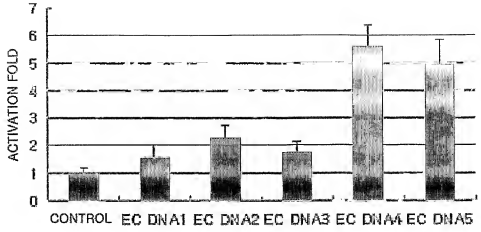


FIG. 1b

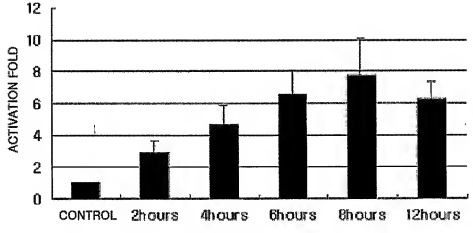


FIG. 2

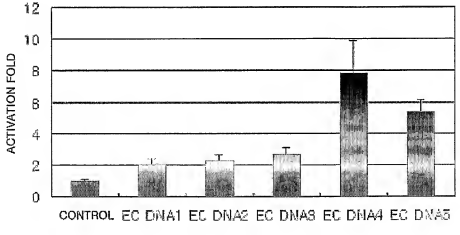
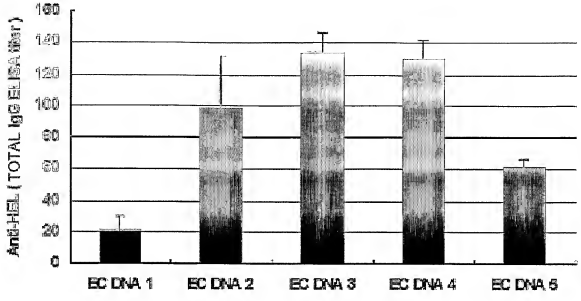


FIG. 3a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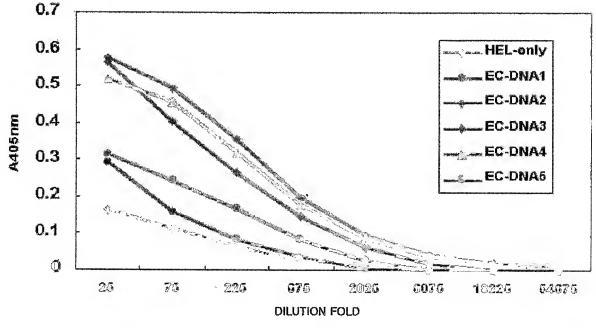
EC DNA 2
EC DNA 3
EC DNA 4
EC DNA 5

FIG. 3b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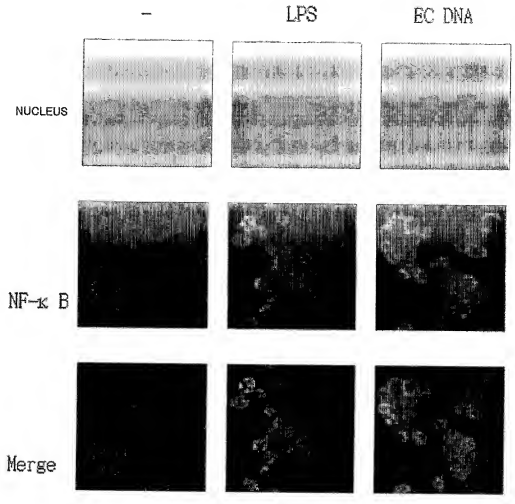
EC-DNA4
EC-DNA6
DILUTION FOLD

FIG. 4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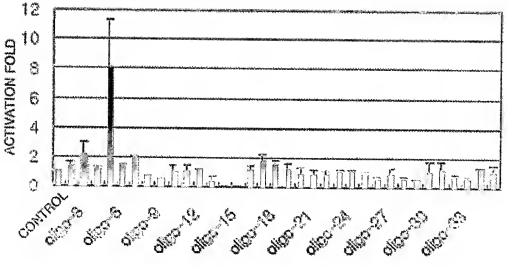
NUCLEUS
NF-κ B
Merge

FIG. 5a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oligo-27
oligo-30
oligo-33

FIG. 5b

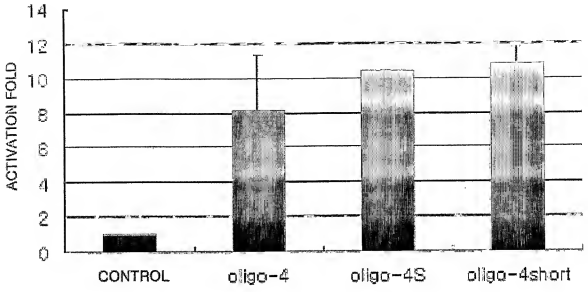
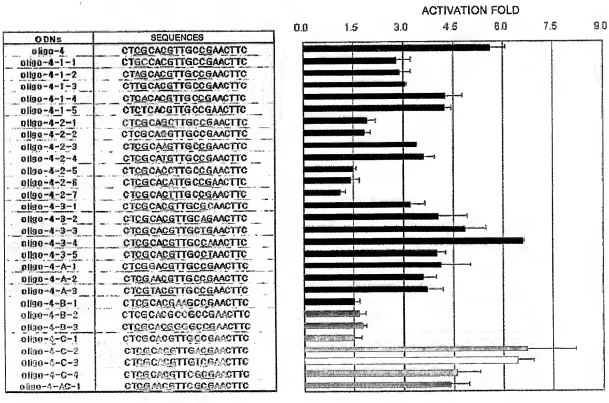


FIG. 6



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oligo-4-2-3	CTCGCAAGTTGCCGAACTTC
oligo-4-2-4	CTCGCATGTTGCCGAACTTC
oligo-4-2-5	CTCGCACCTTGCCGAACTTC
oligo-4-2-6	CTCGCACATTGCCGAACTTC
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oligo-4-3-4	CTCGCACGTTGCCAACTTC
oligo-4-3-5	CTCGCACGTTGCCTAACTTC
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oligo-4-B-2	CTCGCACGCCGCCGAACTTC
oligo-4-B-3	CTCGCACGCCGCCGAACTTC
oligo-4-C-1	CTCGCACGTTCCCGAACTTC
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FIG. 7

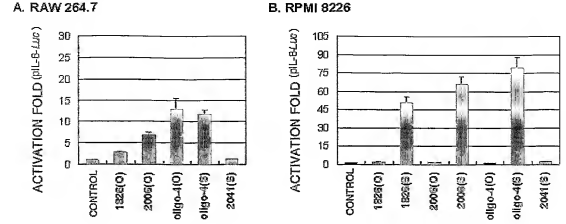


FIG. 8

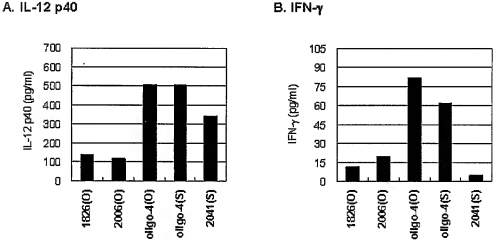
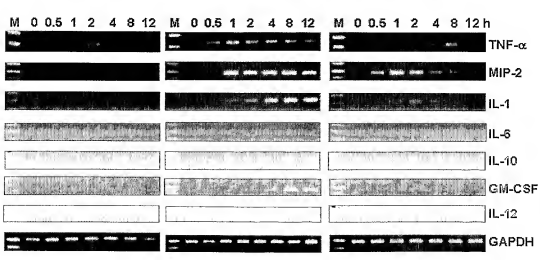


FIG. 9



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INTERNATIONAL SEARCH REPORT

ırnational application No.
PCT/KR2004/000443

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C07H 21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C07H 21/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean patents and applications for inventions since 1975.

Electronic data base consulted during the internationl search (name of data base and, where practicable, search terms used)

STN, Delphion Research Intelletual Propety Network database, NCBI PubMed database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2001/51500 A1 (THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 19 July 2001	1-7
A	US 6218371B1 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 17 April 2001	1-7
A	YU, D., KANDIMALLA, E. R., BHAGAT, L., TANG, J.-Y., CONG, Y., TANG, J., AGRAWAL, S. "Immunomers'-novel 3'-3'-linked CpG oligodeoxtribonucleotides as potent immunomodulatory agents' In Nucleic Acids Research (2002) Vol.30(20):4460-4469	1-7
A	YAMADA, H., GURSEL, I., TAKESHITA, F., CONOVER, J., ISHII, K. J., GURSEL, M., TAKESHITA, S., KLINMAN, D. 'Effect of Supressive DNA on CpG-Induced Immune Activation' In Journal of Immunology (2002) Vol.169:5590-5594	1-7
A	OLBRICH, A. R. M., SCHIMMER, S., HEEG, K., SCHEPERS, K., SCHUMACHER, T. N. M., DITTMER, U. 'Effective Postexposure Treatmnet of Retrovirus-Induced Disease with Immunostimulatory DNA Containing CpG Motifs' In Journal of Virology (November 2002) Vol.76(22):11397-11404	1-7

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 MAY 2004 (21.05.2004)	22 MAY 2004 (22.05.2004)

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
AHN, Kyu Jeong
Telephone No. 82-42-481-5026

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material
      - [X] a sequence listing
      - [ ] table(s) related to the sequence listing

   b. format of material
      - [X] in written format
      - [X] in computer readable form

   c. time of filing/furnishing
      - [X] contained in the international application as filed
      - [X] filed together with the international application in computer readable form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2004/000443

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2001/51500A1	19. 07. 2001	US20030144229 A1	31.07.2003
		EP1322655 A1	24.07.2001
		AU0127889 A5	24.07.2001
US 6218371 B1	17. 04. 2001	WO 1999/51259 A2	14.10.1999
		US20020064515 A1	30.05.2002
		EP1067956 A2	17.01.2001